Published in final edited form as:
Chem Biol Interact. 2013 March 25; 203(1): 85–90. doi:10.1016/j.cbi.2012.10.019.

Proteomic analysis of adducted butyrylcholinesterase for biomonitoring organophosphorus exposures

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Abstract

Organophosphorus (OP) compounds include a broad group of toxic chemicals such as insecticides, chemical warfare agents and antiwear agents. The liver cytochromes P450 bioactivate many OPs to potent inhibitors of serine hydrolases. Cholinesterases were the first OP targets discovered and are the most studied. They are used to monitor human exposures to OP compounds. However, the assay that is currently used has limitations. The mechanism of action of OP compounds is the inhibition of serine hydrolases by covalently modifying their active-site serine. After structural rearrangement, the complex OP inhibitor-enzyme is irreversible and will remain in circulation until the modified enzyme is degraded. Mass spectrometry is a sensitive technology for analyzing protein modifications, such as OP-adducted enzymes. These analyses also provide some information about the nature of the OP adduct. Our aim is to develop high-throughput protocols for monitoring OP exposures using mass spectrometry.

Keywords

Organophosphates; Mass spectrometry; Protein adducts; Butyrylcholinesterase

1. Introduction

Organophosphorus (OP) compounds were first described at the beginning of the 19th century by Jean Pierre Boudet, when he generated traces of ‘phosphoric ether’ as a result of combining alcohol and phosphoric acid [1]. However, it was not until 1848 that Franz Anton Voegeli created the first OP compound, triethyl phosphate. Just 5 years later, Philippe de Clermont described the synthesis of the first OP compound with anticholinesterase properties, the tetraethyl pyrophosphate (TEPP) [1]. In the 1930s, OPs were reported to be toxicants for mammals and insects, a property that triggered military interest and influenced

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Conflict of interest statement: The authors declare that there are no conflicts of interest.

the evolution of OPs into the highly toxic nerve agents sarin, soman and tabun, synthesized by Gerhard Schrader, considered the ‘father of modern OP compounds’ [2]. After World War II, OP compounds saw use as a major class of insecticides (e.g., chlorpyrifos, malathion and parathion), as originally intended by Schrader. In addition to their utility as agriculture chemicals and warfare agents, OP compounds have also been used in industry as plasticizers, flame retardants, fuel additives, and lubricants, or even in medicine as therapeutic agents [3].

OPs are one of the most common causes of poisoning worldwide, with 3 million cases of pesticide poisonings per year, resulting in 220,000 deaths [4]. Their broad use results in many cases in environmental or occupational human exposure, causing a variety of adverse health effects [5]. As a consequence, research on bio-markers for biomonitoring human exposures to OPs has become an area of extensive investigation.

2. Mechanism of action of OP compounds

OPs comprise a large and diverse family of compounds, many of which can be hydrolyzed in the presence of water or by specific enzymes, resulting in detoxification. OP insecticides are mainly derivatives of phosphoric or thiophosphoric acid, containing usually two alkoxy substituent groups and a third substituent known as the ‘leaving group’, as it is displaced when the OP interacts with serine hydrolases [6].

The mechanism of action of OP compounds involves the progressive inhibition of serine hydrolases [7], the main target being acetylcholinesterase (AChE). Their inhibitory action on AChE at nerve synapses seems to be responsible for their toxicity toward both invertebrates and mammals [8]. AChE (EC 3.1.1.7, accession #P22303) is a tetrameric serine esterase present in many tissues, particularly in central and peripheral nervous tissue where it terminates nerve impulse transmission by hydrolyzing the neuro-transmitter acetylcholine at nerve synapses. AChE is also found on red blood cells (RBCs), where its function is unclear. In vertebrates, butyrylcholinesterase (BChE) can also hydrolyze acetylcholine. BChE (EC 3.1.1.8, accession #P06276) is also a tetrameric serine esterase present almost ubiquitously and in plasma. BChE is also inhibited by OP compounds, although this inhibition has no cholinergic symptoms and no known biological effect [8, 9]. The physiological function of BChE is still not clear, although some studies suggest that it could act as an AChE backup [10–12]. This certainly appears to be the case in the AChE knockout mouse generated by Lockridge and colleagues [10]. Furthermore, BChE, as a stoichiometric scavenger, may protect from synthetic and naturally occurring poisons by preventing AChE inhibition by these toxins [13].

3. Biomonitoring OP exposures

Biomonitoring refers to the assessment of human exposure to chemicals and health risk by measuring the chemicals or their metabolites in body fluids, such as blood or urine [14]. Early detection of exposures can enable interventions before severe symptoms occur and follow-up of the recovery of the intoxicated subject.

The term biomarker refers to biological substances that are indicators of exposure or disease [15], and that can be measured by laboratory techniques [16]. There is an increasing interest in identifying and characterizing new biomarkers for a better health assessment.

Blood cholinesterases have long served as biomarkers of OP exposure, since they were the first OP targets discovered. It has been shown that inhibition of RBC AChE can be used as a valuable surrogate for inhibition of neuronal AChE [17, 18], although plasma BChE is more sensitive to some OPs than RBC AChE. Furthermore, both cholinesterases are found in blood, an ideal matrix for bio-marker analysis.

In 1961, Ellman et al. reported a colorimetric enzymatic assay that is currently the standard method for determining occupational or accidental OP exposures [19]. The Ellman assay uses acetylthiocholine or butyrylthiocholine for determining AChE or BChE activity, respectively. Other methods for monitoring cholinesterases have been reported [20], but the Ellman assay has remained the method of choice due to its advantages, being an inexpensive, simple and rapid assay. Despite the advantages, the Ellman assay has also some important drawbacks. It is not an accurate assay at low levels of inhibition (20% or less) [17, 21–23]. OPs causing delayed or chronic toxicity without significant inhibition of cholinesterases cannot be monitored by this method [7, 24]. In addition, it requires a pre-exposure determination of baseline activity level in order to overcome intra- and inter-individual activity variability, and for more accurate assessment of low level inhibition [25, 26]. The pH of the buffers used in the assay and the concentration of substrate can also be critical in obtaining consistent results [20]. Furthermore, the temperature of sample storage is also an important issue. Spontaneous reactivation of the inhibitor-enzyme complex is observed at ambient temperature with some OP inhibitors [20]. OP inhibitions are not irreversible until a structural reorganization, ‘aging’, of the inhibitor-enzyme complex happens (see Section 4). Another limitation is the impossibility of identifying the OP compound responsible for the observed inhibition. Moreover, because the replacement of inactive enzyme by *de novo* synthesized protein can occur rapidly, this method is less amenable for retrospective analyses [22, 23]. When comparing plasma BChE *vs*. RBC AChE, AChE determination can present further disadvantages, resulting from difficulties in reproducible pipetting and washing of the RBCs, or from a potential interference by hemoglobin [20, 27]. In terms of enzyme availability, humans have ten times more BChE than AChE [28], plasma is an easier matrix to handle than RBCs and, as noted above, BChE may be more sensitive to inhibition by some OPs than AChE. Despite these disadvantages, RBC AChE has a longer half-life than plasma BChE (33 *vs*. 11 days, respectively) [29], enabling evaluation of exposures after longer periods of time.

4. OP binding to the active-site serine and proteomics

Inhibition of serine hydrolases by OPs is mainly due to the formation of a covalent bond between the phosphor-alkoxy moiety of the OP compound and the hydroxyl residue of the catalytic serine of the active site of these enzymes, with the resulting release of the OP ‘leaving group’. The OP adduct can suffer further dealkylation through a process called ‘aging’, probably assisted by residues near the active site of the enzyme. Although the initial covalent bond is usually reversible, the aging (loss of one alkoxy group) enhances the

stability of the OP adduct bound to the enzyme, preventing the dissociation of the enzyme-inhibitor complex [12]. The OP-adducted protein will remain in circulation for a length of time dependent on the half-life of the enzyme, generally much longer than the resident time of the OP or its metabolites.

The fact that serine hydrolases are modified by OPs provides another means for identifying the specific OP exposures with the use of liquid chromatography and tandem mass spectrometry (LC–MS/MS) to detect and quantify modified (OP-adducted) proteins. LC–MS/MS is ideal for the identification of a wide variety of protein modifications. It is a highly sensitive and precise technique that can perform accurate mass readings, allowing effective identification of different adducts with similar or identical masses. LC–MS/MS can quantify adducted and non-adducted enzymes, making it suitable for quantitative retrospective analysis. It does not require a baseline activity measurement for the individual, eliminating the need of pre-exposure bleeds. Part of the original OP structure is released during the reaction of the OP with the active-site serine and another part during the aging process; therefore MS may provide ambiguous or limited identification of the specific OP of exposure. LC–MS/MS also has some limitations or disadvantages, such as being time consuming, costly, limited by reactivation, and requiring well-trained personnel [16, 23].

Application of MS to the study of OP exposures was first reported in 2001, using *in vitro* inhibited equine serum BChE [30]. The first '*in vivo*' application came just one year later [31], and since has become a field of intense research [32]. MS has also proven to be useful for the identification of new biomarkers of OP exposure [33]. Both LC and MALDI-TOF MS have been reported to be useful in the identification of OP exposures from either insecticides or nerve agents [21, 33–39]. Most of these studies have focused on BChE, since it is an abundant plasma protein that is easy to obtain and has been extensively studied as a biomarker of OP exposure. The methods described in these studies involve either pure BChE or partial purification of OP-adducted BChE, followed by proteolytic digestion (e.g., pepsin, trypsin or chymotrypsin) and LC–MS/MS or MALDI-TOF analysis. Even though MS provides high sensitivity analyses, plasma is still a very complex matrix and purification of the biomarker protein (or modified peptide) facilitates analysis. One reported partial purification of BChE was achieved by affinity chromatography, which is time consuming, requires specific resins and a large volume of sample, and thus limits the standardization of the assay for high-throughput. Other studies, including those from our laboratory, that also monitor OP exposures by LC–MS/MS, have described the use of immunomagnetic beads coupled to a specific anti-BChE antibody (Fig. 1), a procedure that should overcome the limitations of affinity chromatography for biomarker purification [15, 40–42]. Immunomagnetic separation (IMS) protocols are faster, require smaller quantities of starting material and can be easily adapted for automated high-throughput analysis. The affinity column approach, however, is useful when large quantities of analyte are required to detect a low level of OP adduction.

5. Verification of OP exposures using IMS coupled to LC–MS/MS

In developing analyses for OP-adducted biomarker proteins, our goal was to develop a protocol that (1) used low volumes of plasma, (2) is rapid and easy to perform, (3) is more

sensitive than the Ellman assay, (4) does not require a separate blood draw for determination of baseline activity level, and (5) is easily automated for high-throughput. The main rationale for these goals was the achievement of a rapid procedure that could be used for any event of human exposure resulting in cholinesterase inhibition. Two types of exposure are described below as examples of IMS coupled to LC–MS/MS analyses.

5.1. Aerotoxic syndrome

Tricresyl phosphate (TCP) is an OP compound used as an anti-wear additive in jet engine lubricants and hydraulic fluids [43, 44]. During Prohibition, the *ortho* isomer of TCP was used to adulterate alcoholic ginger extracts, causing thousands of cases of paralysis in the United States [45]. The air supply for the aircraft cabin is directed unfiltered to the aircraft cabin and flight deck from the aircraft engine along the combustion pathway of the engine (referred to as bleed air) [46]. When engine seals wear or fail, significant levels of engine oil fumes can enter the aircraft cabin (fume event). Even under normal operation conditions, some TCP may enter the cabin. For instance, TCP was detected on 23% of the 100 flights monitored in a recent Cranfield University study [47]. It has been estimated (from a survey over an 18-month period) that an average of 0.86 fume events occur per day, although this is suspected to be an underestimation due to underreports of the total number of fume events [46]. During the past 25 years, there have been an increasing number of reports of mainly pilots and flight attendants, experiencing a series of symptoms following fume events, including tremors, dizziness, nausea, disorientation; or long-term numbness, memory loss and fatigue [38, 39, 41]. These symptoms have been referred to as aerotoxic syndrome [48]. Once the TCP enters the human body, it is bioactivated by the liver cytochromes P450 to the toxic molecule, 2-(*ortho*-cresyl)-4*H*-1, 3, 2-benzodioxaphosphoran-2-one, also known as cyclic saligenin cresyl phosphate (CBDP) (Fig. 2) [49]. Although it is unlikely that the symptoms reported above are caused by inactivation of BChE by CBDP, BChE can nonetheless serve as a biomarker of TCP exposure.

Using immunomagnetic beads conjugated with a monoclonal BChE antibody (Fig. 1), we have been able to purify plasma BChE to a high degree of purity starting with 200 μl of plasma or less in only one step [41]. Initially the bound BChE was eluted with acetic acid, digested with chymotrypsin and analyzed by LC–MS/MS. The current protocol avoids the elution step and performs the chymotrypsin digestion on beads, releasing the digested peptides prior to loading the sample to the LC–MS/MS, as depicted in Fig. 1. The rationale for this was to obtain a protocol amenable to automation. The active-site peptide sequence monitored was GESAGAASVSLH (the active-site serine is underlined) [41]. We detected BChE adducted with both cresyl phosphate (+170 Da) and phosphate (+80 Da) adducts on the serine 198 of the active site of *in vitro* exposed samples to CBDP (Fig. 2) [41]. The identification of phosphorylated active-site serine of BChE from passengers on a commercial flight has been reported. However, the amount of modified BChE was low (ranging from 0.05 to 3%) [39]. The limit of detection of the IMS-LC–MS/MS protocol for TCP exposures is about 5%, more sensitive than the Ellman assay, but not yet sensitive enough to detect very low levels of exposures. We are currently focusing on identifying more sensitive biomarkers for monitoring TCP exposures.

5.2. Agricultural workers

OP insecticides are widely used in agriculture. Despite the drawbacks described above, the Ellman assay is currently the standard method used for monitoring OP insecticide exposures. In the United States, the states of California and Washington have ongoing monitoring programs where agricultural workers at high risk of OP insecticide exposure are tested for both AChE and BChE inhibition prior to and during the work/spray season. The use of the Ellman assay for monitoring their cholinesterases requires obtaining a pre-season bleed so they can compare their cholinesterase basal levels with their levels during the work season [50, 51]. If their cholinesterase levels decrease 40% or more from the basal activity levels, workers are removed from the exposure environment, without loss of wages, until their cholinesterase levels return to 80% of their basal level. The goal of these mandatory monitoring programs is to protect the health and safety of the agricultural workers.

The use of MS for monitoring these agricultural workers should overcome many of the limitations of the Ellman assay. We have used the following improved version of the IMS coupled with the LC–MS/MS protocol detailed above where 100 μl of plasma is incubated with immunomagnetic beads conjugated with a monoclonal BChE antibody. Following washing of the BChE/bead complex, an on-bead digestion of the bound BChE with chymotrypsin is carried out followed by LC–MS/MS analysis of the peptide containing the active-site serine 198. Our goal was to monitor a cohort of Washington State agricultural workers and compare the results with the Ellman assay inhibition measurements of the same workers.

The preliminary *in vitro* experiments focused on characterizing BChE active-site serine adducts resulting from chlorpyrifos oxon (CPO) and azinphos methyl oxon (AZO) inhibition; the toxic oxon metabolites of two commonly used OP insecticides. The same active-site dodecapeptide described above was obtained from the chymotrypsin digestion of BChE. We were able to identify the diethyl phosphoserine and mono-ethyl phosphoserine adducts following exposure to CPO, which revealed a gain of 154 or 126 Da compared to the unmodified BChE active-site peptide, respectively (Fig. 3A). Following incubation with AZO, the analyzed BChE active-site peptides revealed di-methyl phosphoserine (gain of 126 Da) and mono-methyl phosphoserine (gain of 112 Da) adducts on serine 198 (Fig. 3B). The limit of detection of this method is about 1–2%.

We are currently carrying out LC–MS/MS analysis of plasma samples from 128 agricultural workers, whose BChE activity inhibition levels are known. The preliminary data indicate exposure of some agricultural workers to CPO (*vs*. AZO) (Fig. 4). No AZO exposure was identified during our preliminary analyses. The IMS purification coupled with LC–MS/MS analysis of adducted BChE peptides correlated well with the Ellman activity inhibition assay; however more analyses need to be carried out to corroborate these findings. With some of the analyzed samples, the Ellman assay reported a gain of activity compared with the basal measurement of BChE activity, while the LC–MS/MS identified either no adducts or adducts indicating low levels of CPO exposure (data not shown).

6. Conclusions and future perspectives

Early detection of OP exposures can allow for clinical interventions before severe symptoms occur and an improved follow up of the recovery of the subject. A 50% inhibition of neural AChE or greater is necessary for developing anti-cholinergic symptoms [52]. The LC–MS/MS analysis of OP modified BChE has several advantages over the currently used colorimetric enzyme activity assays. The analysis does not require a separate blood draw for the determination of baseline activity levels, it provides a more accurate measurement of the percentage modification of the active-site serine, and it also provides some information about the OP that adducted the active-site serine (e.g., a methyl- or ethyl-based insecticide).

The focus on the use of MS analysis for biomonitoring exposures to OP compounds represents a significant advantage for the identification and characterization of biomarkers of exposure. In recent years, there have been important improvements in MS technology, paving the way for the use of these analyses in standard clinical laboratories. Some of the current limitations of MS, including costs of analyses, time required for analyses, and the requirement for highly trained workers may be overcome in the near future.

Acknowledgments

This research by the authors was supported by the following Grants from the National Institutes of Health: 5P42ES04696, NIEHS P30ES07033, R01ES09883, ES09601/EPA-R826886, P41GM103533; and by financial support from the Centers for Disease Control and Prevention (CDC, Atlanta, GA).

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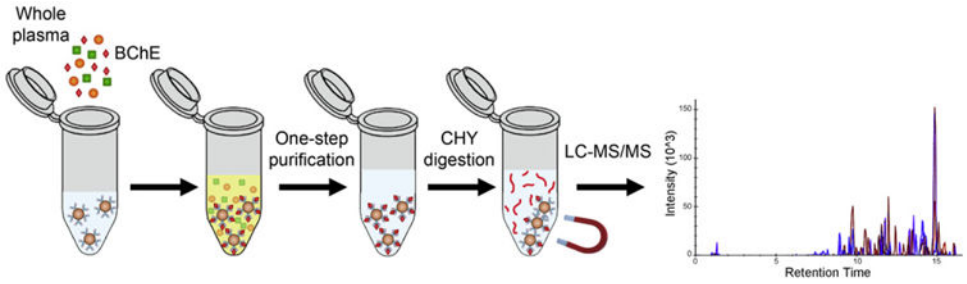

Fig. 1.

Immunomagnetic bead separation of BChE coupled to LC–MS/MS analysis. This protocol efficiently purifies human BChE from 100 μl of plasma or less in one single step, and is readily adaptable for high-throughput analytical protocols. Samples are digested on-beads with chymotrypsin (CHY) and analyzed by LC–MS/MS.

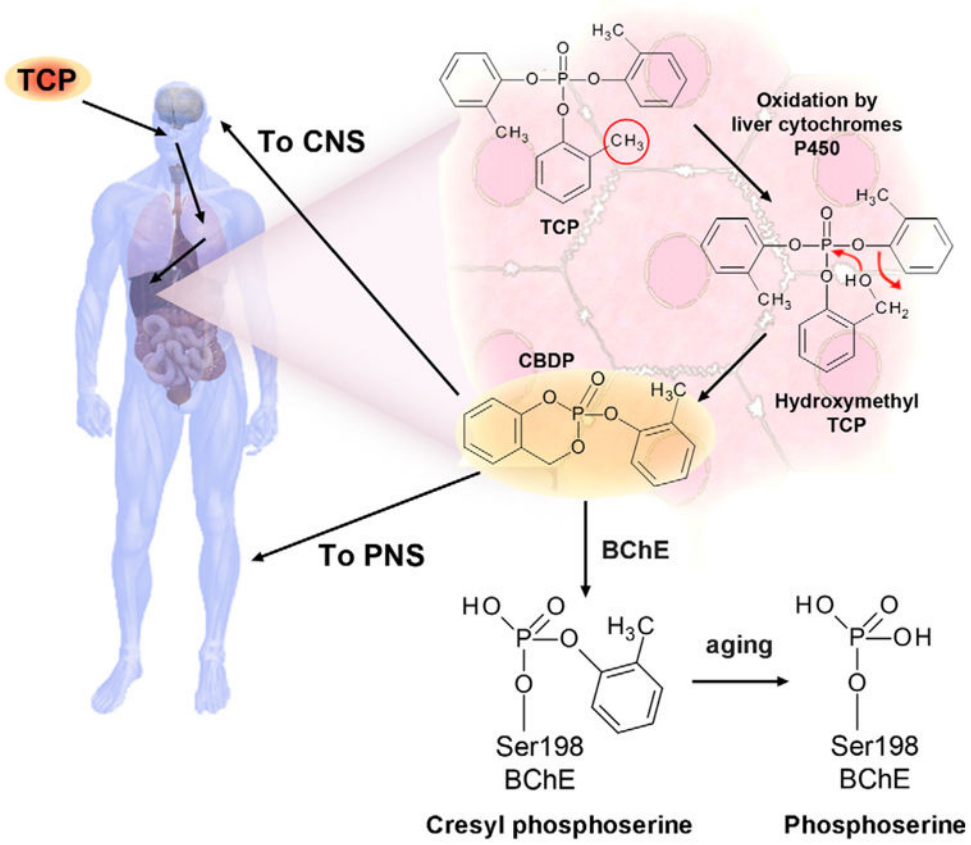

Fig. 2.

Bioactivation of tricresyl phosphate (TCP) to the highly toxic metabolite cyclic saligenin cresyl phosphate (CBDP) by the liver cytochromes P450. CBDP affects both the central (CNS) and peripheral nervous system (PNS). CBDP inhibits BChE yielding a cresyl phosphate on the serine 198 of the active site that ages to phosphoserine.

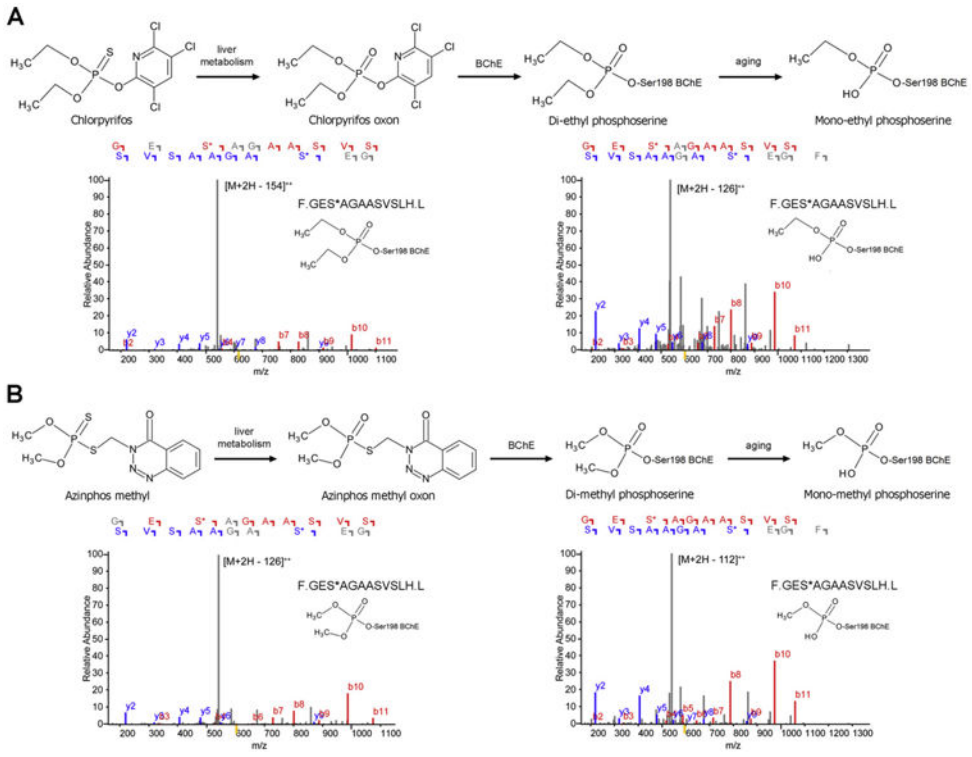

Fig. 3.

OP insecticides and MS analysis using *in vitro* inhibited BChE. (A) Metabolism, BChE adduction and aging of chlorpyrifos. The MS analysis of the BChE active-site peptide detected both adducts, the di-ethyl phosphoserine and the aged mono-ethyl phosphoserine. (B) Metabolism, BChE adduction and aging of azinphos methyl. The MS analysis of the BChE active-site peptide detected both adducts, the di-methyl phosphoserine and the aged mono-methyl phosphoserine.

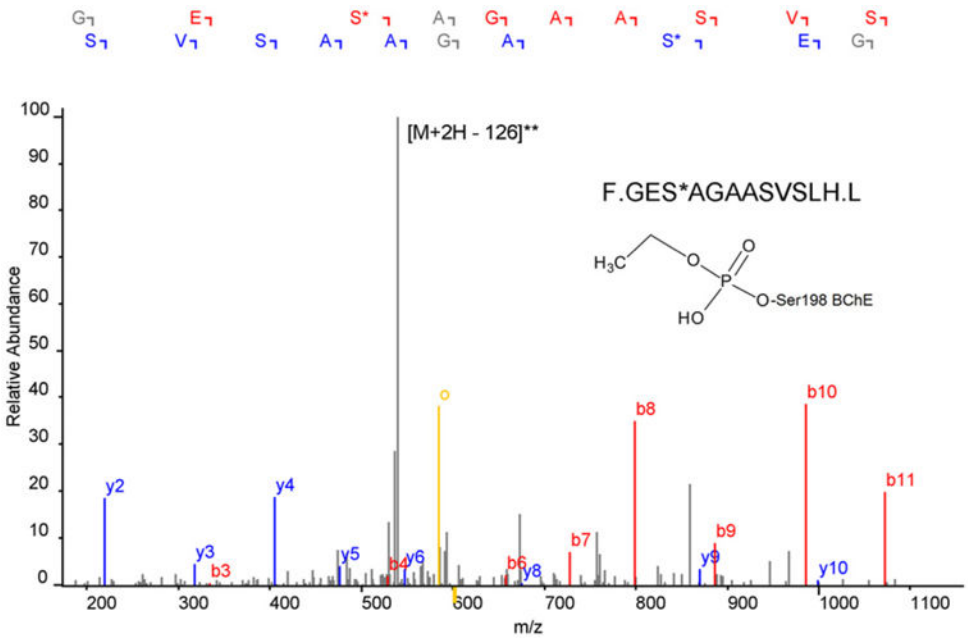

Fig. 4.

Identification of chlorpyrifos exposure in plasma of an agricultural worker. The adduct on the active-site serine corresponds to the aged mono-ethyl phosphate.